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14. ABSTRACT
We seek to design and implement a genetic circuit that based on multiple genetic markers is able to selectively recognize and destroy cancer cells, leaving healthy cells unaffected. In this project we focus on the MCF-7 breast adenocarcinoma cell line, a well-characterized cell line derived from a common form of breast cancer. MCF-7 cells overexpress Gata3, NPY1R and TFF1 mRNA relative to healthy cells. Based on our bioinformatics analysis, taking into account the three biomarkers allows for dramatically improved specificity in comparison to targeting single genes. We therefore design our circuit so that it only targets for destruction cells with high levels of mRNA of all three biomarkers (an AND gate).
We have tested and successfully implemented each of the necessary circuit components: very efficient siRNA and microRNA gene knockdown, hBax dependent apoptosis, expression of mStaple (a short regulatory mRNA), and non-integrating lentivirus. Our current efforts are focused on careful characterization of the modules as a preliminary step for final implementation and fine-tuning of the full circuit. In the next step we will introduce all of the circuit components into HEK 293 and MCF-7 cells.

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INTRODUCTION

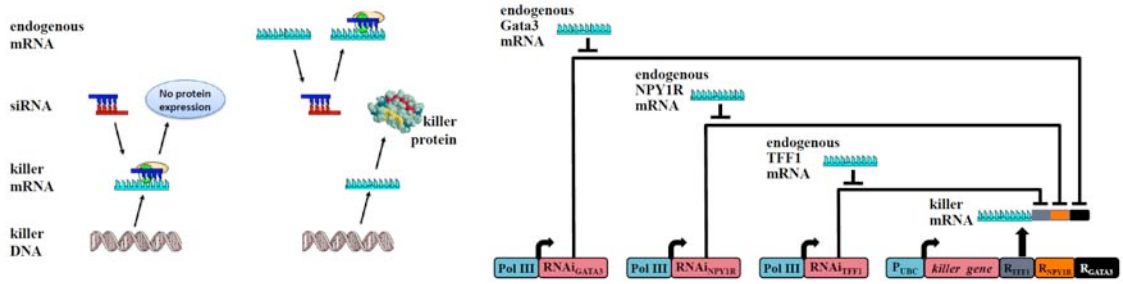
Objective

We seek to design and implement a genetic circuit that based on multiple genetic markers is able to selectively recognize and destroy cancer cells, leaving healthy cells unaffected. In this project we focus on the MCF-7 breast adenocarcinoma cell line, a well-characterized cell line derived from a common form of breast cancer. MCF-7 cells overexpress Gata3, NPY1R and TFF1 mRNA relative to healthy cells. Based on our bioinformatics analysis, taking into account the three biomarkers allows for dramatically improved specificity in comparison to targeting single genes. We therefore design our circuit so that it only targets for destruction cells with high levels of mRNA of all three biomarkers (an AND gate).

BODY

I. Approach

We investigate two versions of the circuit design. The two approaches share many common components, but differ moderately in the way the cell state is being evaluated.
In approach I (Figure 1), three different shRNA are expressed, processed by Dicer to siRNAs and subsequently target the three chosen biomarkers of cancer (cell state evaluator module of the circuit). The target sites for the siRNAs are also artificially attached in the 3'UTR of the killer/reporter gene. When all the siRNA molecules are titrated away by the targeted mRNA molecules (high biomarker levels, cancer cells), the killer gene is expressed and leads to apoptosis. If any of the biomarkers is expressed at low level, the corresponding siRNA targets 3'UTR of the killer gene, resulting in cell survival. One of the potential problems in this approach is that the mRNA of biomarkers is targeted by siRNA also in non-cancerous cells and that may affect cells health. Also, there is no energetical preference for siRNA binding to the biomarker mRNA over the killer mRNA, and that may lower the killing efficiency even in cancer cells.

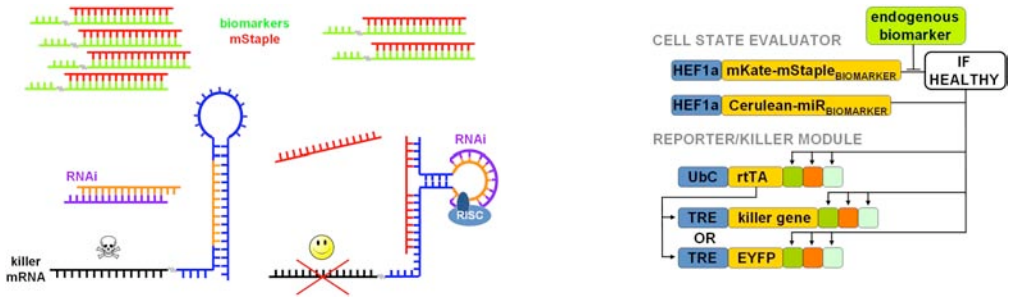


(a) (b) (c)

Figure 1: RNAi logic circuit-based approach I. (a-b) Killer protein (e.g. Bax) expression depends on levels of endogenous marker mRNA as mediated by siRNA interactions. (c) For the 3-input AND gate, the endogenous levels of Gata3, NPY1R and TFF1 all need to be high in order to titrate away the three engineered siRNAs and allow expression of the killer protein.

In the second approach (Figure 2), the components of our proposed circuit also include an apoptotic gene with an engineered regulatory sequence (RS), short interfering RNA (siRNA) directed against the RS, and a set of additional short mRNA sequences, mStaples. Each mStaple molecule is complementary to a specific cancer biomarker and partially complementary to a portion of the RS. The role of mStaple is to regulate siRNA mediated degradation of the apoptotic gene. In the absence

of mStaple, the RS forms a stem loop where the siRNA binding site is hidden and does not allow for siRNA binding and degradation of the mRNA. As a result, the cell undergoes apoptosis. When the mStaple binds to the RS, it enforces a conformational change of the sequence and exposes siRNA binding site. The mRNA of the apoptotic gene is degraded and the cell survives. The expected behavior is therefore abundance of the mStaple in normal cells and it's shortage in cancer cells. In our system the mStaple is expressed similarly in all cell types, but it's availability for binding of the RS depends on the level of endogenous genes – cancer biomarkers. The mStaple binds preferentially to the biomarker mRNA and with lower affinity to the RS. In normal cells with low biomarker levels some of the mStaple will be bound by the biomarker and some will target the RS to expose the siRNA binding sequence. In cancer cells, when the biomarker level is high, the mStaple will be titrated away, causing no disruption in expression of the apoptotic gene, and ultimately cell death.



(a) (b) (c)

Figure 2: RNAi-based logic circuit, approach II. Similarly as in approach I, killer protein is dependent on the endogenous biomarker mRNA. For testing purposes, we replace the killer gene with a reporter, EYFP gene in the first implementation of the circuit.

Both circuit designs require efficient siRNA mediated knockdown and testing of the pro-apoptotic genes. Controllable expression of the short mStaple sequence is also needed for the second approach. We will address progress on each of these modules in the next section.

II. Results

II.a. Circuit design and modeling

We have carefully designed the regulatory sequence (RS) at the 3'UTR of the killer/reporter gene to program a conformational change of the RS in the presence of mStaple molecules. DNA and RNA structures are much easier to predict and design than protein structures and the folding rules are well established in the DNA origami field. The mStaple molecule binds to the Gata3 gene with perfect complementarity and it is only partially complementary to the designed regulatory sequence. Therefore, it will bind to the killer/reporter gene 3'UTR with lower affinity, and preferentially only after all Gata3 binding sites have been satisfied. Upon mStaple binding, the RS mRNA fragment undergoes a conformational change, exposing siRNA binding site. Such design allows for Dicer mediated degradation of the killer/reporter mRNA in cells with low biomarker levels. In Figure 3 we show predicted structures of a single biomarker, Gata3-dependent regulatory sequence alone (A) and in the presence of mStaple (B).

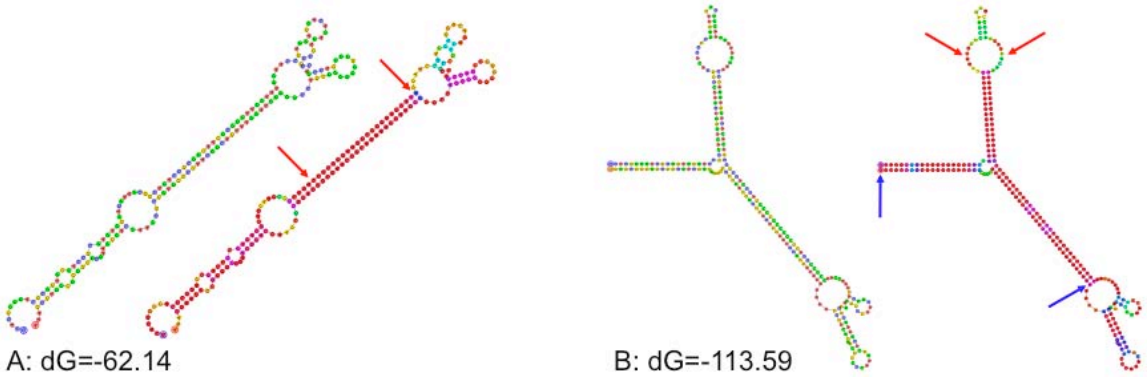


Figure 3: Secondary structure prediction for Gata3-dependent regulatory sequence with mfold [1,2]. Left panels are color coded according to nucleotide composition, and right panels according to structure formation probability (red indicates high). In the absence of mStaple (A), siRNA target site (within red arrows) is hidden by perfect base-pair complementarity. To simulate the presence of mStaple, its sequence has been added to the regulatory element sequence with a (polyA)6 linker (B). Upon mStaple binding (blue arrows), a more stable structure is formed and the siRNA target site gets exposed.

II.b. RNAi knockdown

We have tested multiple siRNA molecules by placing the corresponding target site sequences in the 3'UTR of the EYFP gene and co-transfecting the 293FT cells with the EYFP-target construct and siRNA or plasmid expressing shRNA. Example siRNA knockdown results (FF4 siRNA [3]) are presented in Figure 4. Similar knockdown efficiency was also achieved with two other siRNA molecules, FF3 and FF6 [3]. All of the tested siRNA molecules target the *Renilla firefly* luciferase and are completely orthogonal to the mammalian expression system.

In the next step we tested knockdown efficiency, when siRNA is expressed from a transfected plasmid and then processed in the cell to its mature form. FF4 shRNA was placed in the miR-30 microRNA backbone and the construct was expressed as an intronic sequence within a cyan fluorescent protein, AmCyan [3]. The miR-FF4 intron sequence introduces three in-frame STOP codons in the AmCyan sequence, thus the fluorescent protein can only be produced, when splicing occurs.

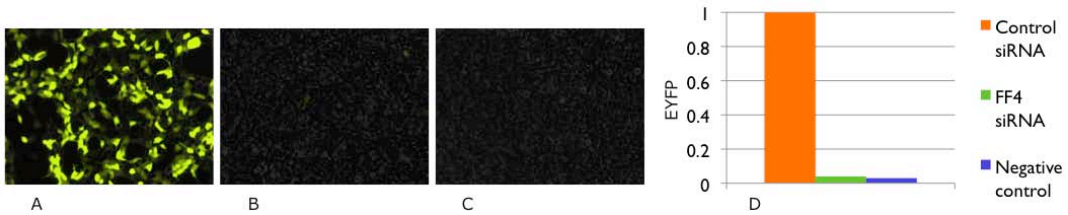


Figure 4: 293FT cells were co-transfected with TRE-EYFP-4xFF4 plasmid and (A) 5 pmol of control siRNA or (B) 5 pmol of FF4 siRNA. (C) negative control, non-transfected 293FT, (D) corresponding normalized FACS results.

As shown in Figure 5, the amounts of fluorescence produced from AmCyan and AmCyan-miR-FF4 constructs are comparable and miR-FF4 is a very potent inhibitor of EYFP-4xFF4. As quantified by qPCR, the knockdown efficiency is 98%.

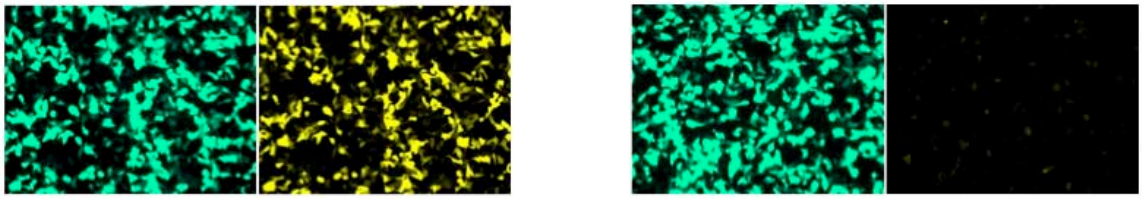


Figure 5: 293FT cells were co-transfected with 200ng of TRE-EYFP-4xFF4 and 600 ng of (A) Hef1a-AmCyan or (B) Hef1a-AmCyan-miR-FF4 constructs. Left panels show cyan channel, and right panels, the yellow channel of the fluorescent microscope images.

We additionally verified splicing by extracting total cell mRNA from cells infected with Hef1a-AmCyan and Hef1a-AmCyan-miR-FF4 constructs. The mRNA was later reverse transcribed to cDNA and PCR with AmCyan specific primers was performed. The gel image is shown in Figure 6.

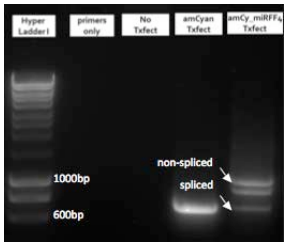


Figure 6: PCR with AmCyan specific primers on the total cDNA cell extract from 293FT cells transfected with Hef1a-AmCyan and Hef1a-AmCyan-miR-FF4.The expected fragment sizes are 1083bp (no splicing), and 707bp (splicing occurs). The size shift visible on the gel confirms splicing of the intron. The three different fragments for AmCyan-miR-FF4 transfected cells were extracted and sequence-verified. The middle fragment contained parts of spliced and non-spliced sequences; this is most likely a by-product of the self-catalytic splicing reaction.

II.c. Bax induced apoptosis

In the final design of our circuit, a reporter gene will be replaced with a pro-apoptotic protein. For that purpose we have explored a few possibilities including diphtheria toxin, a monomeric protein secreted by *Corynebacterium diphtheriae*, and pro-apoptotic members of Bcl-2 family: mBax (Mus musculus), hBax (Homo sapiens), and its mutant hBax-S184A [4]. A plasmid containing the tested gene was transfected into HEK 293FT cells and cell death was monitored by comparison with cells transfected with an equal amount of a plasmid expressing EGFP. The pro-apoptotic gene-containing construct was either cotransfected with an EGFP expressing plasmid (diphtheria toxin and mBax) or in the case of hBax, expressed as an hBax-EGFP fusion. The diphtheria toxin appeared to be too potent and its expression is not tolerated with any promoter leakiness. The mBax, on the other hand, did not exhibit sufficient potency and only after 4 days of expression some cell toxicity was observed. The hBax and its mutant seem to be the best choices, as they induce apoptosis quickly and strongly, and potency can be regulated by mutagenesis [4] or compensated by co-expression of cell survival promoting proteins such as Bcl-2 or Bcl-X_L. The results showing hBax induced apoptosis are presented in Figure 7.

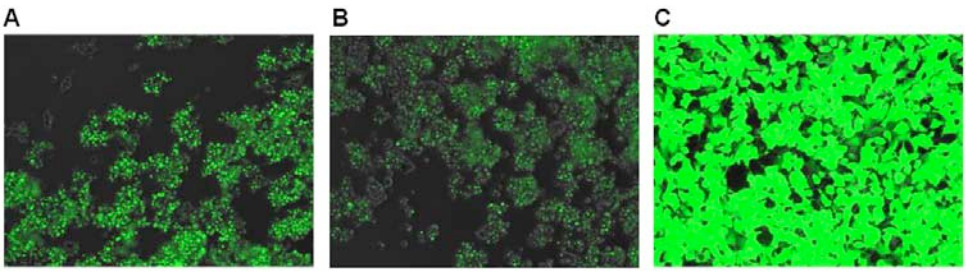


Figure 7. hBax induced apoptosis: 293FT HEK cells were imaged 48h post transfection with constructs constitutively expressing (A) EGFP-hBax fusion protein, (B) EGFP-hBax-S184A fusion, (C) and EGFP alone.

II.d. mStaple expression

We designed a disruptive artificial intron in the coding sequence of the far-red fluorescent protein mKate to express the Gata3 mStaple. Intron-feature sequences – donor site, branch point, poly-pyrimidine tract, and acceptor site – were selected based on previously reported works. The donor site begins the 5' exon-intron junction, and the acceptor site ends the 3' intron-exon junction. Among the donor and acceptor sequences found in literature our intron features were chosen according SplicePort [5], an online analyzer that detects the likelihood of splicing to occur at a specific donor and acceptor site given the sequence context. The final Gata3 mStaple intron design was 5'-GTAAGTGGTCCAAAGGACAGGCTGGATGGCGGGTGCATCGGCGTGGGCGTGGTACTAACTTAACTCGAGTCTTCTTTTTTTTTTTTCACAG-3', the features of which were donor site, Gata3 mStaple, branch point, an in-frame stop codon, XhoI cut site, poly-pyrimidine tract, and acceptor site, respectively. The 96bp Gata3 mStaple intron sequence was inserted into mKate between positions 271 and 272 via PCR.
Similarly as in the case of AmCyan-miR-FF4, the fluorescent mKate protein can only be produced when splicing does occur. In Figure 8 we show FACS results for cells infected with Hef1a-mKate, Hef1a-mKate-Gata3intron, and Hef1a-mKate-Gata3intron-21 constructs. The last construct is similar to Hef1a-mKate-Gata3intron, but includes additional 21bp sequence between the branch point and the acceptor site. Gata 3 mStaple intron is relatively short compared to typical intronic sequences, and we wanted to test if the intron size increase has any effect on the splicing efficiency.

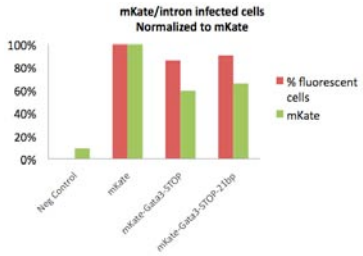


Figure 8: FACS results for 293FT cells infected with constructs expressing mKate (Hef1a-mKate) or Gata3 mStaple as an intron within mKate sequence (Hef1a-mKate-Gata3intron, Hef1a-mKate-Gata3intron-21 constructs). Fluorescence levels for the intronic constructs suggest 60-70% splicing efficiency.

Based on the FACS data, the splicing efficiency is about 60-70%. To finally confirm that no fluorescence can be produced from the non-spliced intronic mKate mRNA, we also created two additional constructs: Hef1a-half-mKate containing only the first exon of the protein and Hef1a-mKate-STOP, that has
a STOP codon in place of the intronic sequence. As expected, transfection of 293FT cells with these constructs produced no fluorescence.
We will additionally verify the splicing efficiency by qPCR.
The cells expressing Gata3 mStaple display normal morphology and viability.
We will perform additional tests (i.e. interferon response activation, qPCR of endogenous Gata3 levels) to verify that expression of the mStaple is indeed neutral to the cells.

II.e. Non-integrating lentivirus

To eliminate insertional mutagenesis but still allow circularization of the viral genome for packaging, reverse transcription after infection, and nuclear import of the preintegration complex, we will use a type I lentiviral mutant integrase. Non-integrating lentivirus is a promising candidate for efficient gene delivery due to its retention of desirable virus activities like packaging and reverse transcription without the risk of non-specific integration into the host DNA [6]. Integration of the viral DNA is mediated by the 32kd viral integrase (IN) which is encoded by the pol gene [6]. Integrase catalyzes the reaction of 3'-OH groups at the viral DNA ends attacking the phosphodiester bonds on the host DNA by several mechanisms [7]. The D64-D116-E152 catalytic triad in the IN core domain is highly conserved and essential for integration. Residue Q148 is involved in binding to the viral DNA at the

U3 and U5 sites of long terminal repeats, whereas N120 and W235 are responsible for binding to the host DNA [6]. Acetylation of three lysines in the C terminus, K264, 266, and 273, enhance viral strand transfer activity by increasing the binding affinity to the host DNA [8]. A number of past studies have shown that point mutations of these crucial residues have led to various degrees of integrase deficiency. For our proposal, we introduce a combination of these point mutations to generate class I mutants that are deficient in integration but not in assembly or reverse transcription [7]. A mutant of IN was created and sequenced: D64V + N120L + W235E.
To evaluate the expression of eGFP over time, HEK-293FT cells were infected with UbC-EGFP packaged with the IN mutant as well as wild-type IN. Flow cytometry was used to measure EGFP expression for 11 days after infection with n = 3 for each vector. Cells infected with the D64V + N120L + W235E mutant IN vector showed an 18-fold reduction in EGFP expression compared to wild-type at 11 days after transduction, comparable to background levels (Fig. 9). Transduction of EGFP expression by wild-type integrase actually increased over the course of the experiment, indicating successful integration in these dividing cells. We will use the mutant integrase to deliver the circuit transiently to all cells. Successful circuit operation in the particular cancer cell line that we target activates the apoptotic pathway even with a mutant integrase whereas other cell types are not affected by circuit operation; ultimately this circuit is degraded and diluted with no permanent effects.

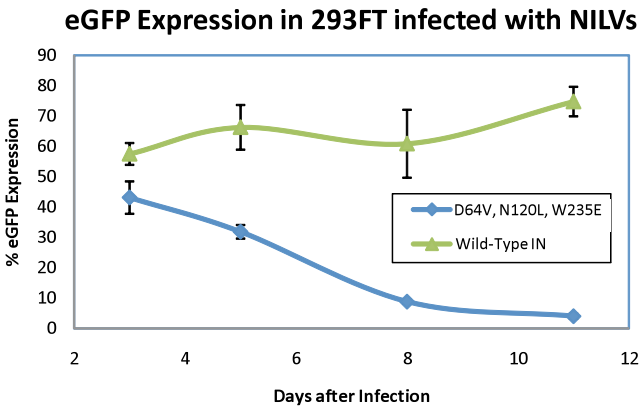


Figure 9: Experimental data for non-integrating lentivirus demonstrating how GFP expression from a constitutive promoter is initially high using both a wild type integrase and a mutant, while over the course of 10 days GFP intensity is reduced to essentially auto-fluorescence levels for the NI-lentivirus.

III. Outreach

The following students were involved in the project and mentored:
Genia Dubrovsky (junior project, 2009)
Anna Igorevna Podgornaia (rotation student, 2009)
Hattie Chung (Amgen scholar, present)

KEY RESEARCH ACCOMPLISHMENTS

- Design and theoretical prediction of a novel mRNA binding based mechanism for distinguishing cells in different states (manifested by different mRNA expression levels);
- Identification of highly efficient siRNA sequences orthogonal to mammalian expression system;
- Implementation of stably integrated microRNA silencing circuit in HEK 293FT cells;
- Design and implementation of splicing-based expression of a short regulatory mRNA: mStaple;
- Construction of a non-integrating lentivirus for safe vector delivery.

REPORTABLE OUTCOMES

Within the scope of the research we have created the following stable cell lines:

HEK 293FT:TRE-EYFP-4xFF4 (microRNA-dependent TetON system)

HEK 293FT:TRE-EYFP-4xT1 (microRNA-dependent TetON system)

HEK 293FT:UbC-rtTA3-4xFF4 (microRNA-dependent TetON system)

HEK 293FT:TRE-EYFP-4xFF4:UbC-rtTA3-4xFF4 (microRNA-dependent TetON system)

HEK 293FT:TRE-EYFP (TetON system)

HEK 293FT:UbC-rtTA3-2A-Hygromycin (TetON system)

HEK 293FT:TRE-EYFP-4xFF4:UbC-rtTA3-2A-Hygro (microRNA-dependent TetON system)

HEK 293FT:Hef1a-AmCyan-miRFF4 (microRNA expression)

HEK 293FT:Hef1a-AmCyan

HEK 293FT:CMV-Bcl2-UbC-Phleomycin (Bax/Bcl apoptotic system)

HEK 293FT:Hef1a-mKate-Gata3intron (splicing dependent mStaple expression)

Glossary of terms:

CMV, Hef1a, UbC – constitutive promoters

TRE – tetracycline response element, inducible promoter

rtTA3 – transcription factor, TetON system

EYFP, AmCyan, mKate – yellow, cyan and red fluorescent proteins

Hygromycin, Phleomycin – antibiotic resistance markers

CONCLUSION

We have designed, tested and successfully implemented each of the necessary circuit components: very efficient siRNA and microRNA gene knockdown, hBax dependent apoptosis, mStaple expression, and non-integrating lentivirus. Our current efforts are focused on careful characterization of the modules as a preliminary step for final implementation and fine-tuning of the full circuit. In the next step we will introduce all of the circuit components into HEK 293 and MCF-7 cells. We will also develop a computational model of the circuit to guide the optimization of the circuit components.

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